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Controlling Protein Conformations to Explore Unprecedented Material Properties by Single-Molecule Surgery

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Hong Peter Lu

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14. ABSTRACT
The primary objective of our single-molecule material science project is to mechanically and optically control and regulate single-molecule protein conformations to explore unprecedented properties and capture such exclusive states in real-time at an extreme molecular sensitivity. Bring up never-detected and novel properties from proteins, over the last year, we have (1) revealed a time bunching effect of protein conformational motions under enzymatic reactions, which suggests that new material properties can be selectively induced and intensified under specific

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a. REPORT	b. ABSTRACT	c. THIS PAGE			H. Lu
UU	UU	UU	UU		19b. TELEPHONE NUMBER: 419-372-1840

Report Title

Controlling Protein Conformations to Explore Unprecedented Material Properties by Single-Molecule Surgery

ABSTRACT

The primary objective of our single-molecule material science project is to mechanically and optically control and regulate single-molecule protein conformations to explore unprecedented properties and capture such exclusive states in real-time at an extreme molecular sensitivity. Bring up never-detected and novel properties from proteins, over the last year, we have (1) revealed a time bunching effect of protein conformational motions under enzymatic reactions, which suggests that new material properties can be selectively induced and intensified under specific conditions identified by single-molecule experiments. The unprecedented properties can only be observed and studied by our single-molecule approaches; (2) discovered, for the first time, oscillatory enzyme conformational motions that are critical for the enzyme activity, which shed light on developing new material technology to induce, intensify, and lock random and transient material properties; (3) demonstrated a manipulation of protein conformations by force pulling a specific residue of a target protein, achieving a new technical milestone towards our goal of manipulating single-molecule proteins to create new material properties; (4) explore matrix magnetic tweezers manipulation of protein activities in enzymatic reaction and protein sensing; and (5) identified a generally existed, but hidden for conventional measurements, time bunching effect of protein conformational motions under enzymatic reactions.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

H. P. Lu, “Single-Molecule Protein Conformational Dynamics in Enzymatic Reactions and Cell Signaling,” Medical School and Department of Chemistry, University of Southern California, Los Angeles, Sept. 25-28, 2008.

H. P. Lu, “Single-Molecule Protein Conformational Dynamics in Enzymatic Reactions and Cell Signaling,” Department of Chemistry, Boston College, Boston, Nov. 5, 2008.

H. P. Lu, “Probing Single-Molecule Protein Conformational Dynamics in Enzymatic Reactions,” Single-Molecule Biophysics Meeting, Aspen, CO, Jan. 4-10, 2009.

H. P. Lu, “Single-Molecule Protein Conformational Dynamics,” Department of Physics and Department of Chemistry, Arizona State University, Tempe, Apr. 8, 2009.

H. P. Lu, “Single-Molecule Protein Dynamics in Enzymatic Reactions and Cell Signaling,” Single Molecule Dynamics International Workshop, Telluride, CO, June, 22-26, 2009.

H. P. Lu, “Revealing Bunching and Memory Effects in Single-Molecule Protein Conformational Dynamics under Enzymatic Reactions,” the 239th ACS National Meeting, San Francisco, Mar. 21-25, 2010.

H. P. Lu, “Manipulating Single-Molecule Protein Conformations for Novel Dynamics,” Single Molecule Dynamics International Workshop, Telluride, CO, June, 13-17, 2010.

H. P. Lu, “Revealing Bunching and Memory Effects in Single-Molecule Protein Conformational Dynamics under Enzymatic Reactions,” the 240th ACS National Meeting, Boston, Aug. 22-26, 2010.

H. P. Lu, “Revealing Bunching and Memory Effects in Single-Molecule Protein Conformational Dynamics under Enzymatic Reactions,” the 242th ACS National Meeting, Denver, Aug. 27-31, 2011.

H. P. Lu, “Probing Single-Molecule Enzyme Active-Site Conformational State Intermittent Coherence and Time Bunching Dynamics,” the 243th ACS National Meeting, San Diego, Mar. 25-29, 2012.

H. P. Lu, “Probing Single-Molecule Enzyme Active-Site Conformational State Intermittent Coherence and Time Bunching Dynamics,” the APS Regional Meeting, Columbus, Apr. 13-14, 2012.

H. P. Lu, “Probing Single-Molecule Enzyme Active-Site Conformational State Intermittent Coherence and Time Bunching Dynamics,” Characterizing Landscapes: From Biomolecules to Cellular Networks International Workshop, Telluride, CO, June, 11-15, 2012.

Number of Presentations: 12.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received	Paper

TOTAL:

Number of Manuscripts:

Books

Received	Paper	
08/17/2012	8.00	H. Peter Lu. Single-Molecule Protein ConformationalDynamics in Enzymatic Reactions, New York, NY: Springer Publishing, (07 2010)

TOTAL: **1**

Patents Submitted

None

Patents Awarded

None

Awards

Olscamp Research Award, BGSU, 2009

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Suneth Rajapaksha	0.50	
FTE Equivalent:	**0.50**	
Total Number:	**1**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Yufan He	0.50
Yuanmin Wang	0.20
Desheng Zheng	0.30
FTE Equivalent:	**1.00**
Total Number:	**3**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
H. Peter Lu	0.06	
FTE Equivalent:	**0.06**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Suneth Rajapaksha
Total Number: 1

Names of other research staff

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See Attachment for details

The primary aim of our single-molecule material science project is to bring up never-detected and unprecedented properties from proteins. If a random and transient (fluctuating) material property, typically non-detectable and lost as it is spatially and temporally averaged out, can be induced to appear at a specific temporal or frequency domain, then a novel property is created from the protein material. During the four years of the project, we have made significant progresses towards realizing the aim of our project. Specifically, we have made discoveries of a novel bunching effect and a coherence dynamics of protein conformational properties that are critical for enzyme functions. We have provided a novel approach to create unprecedented material properties by inducing the specific properties bunched and/or coherence in time. We have also made a significant technical development on demonstrating a manipulation of protein conformations by force pulling a specific residue of a protein, accomplishing a technical milestone of manipulating single-molecule proteins to create new material properties. We have already published a number of papers to report our discoveries, including a recent article published in J. AM. Chem. Soc. on "Probing Single-Molecule Enzyme Active-Site Conformational State Intermittent Coherence," and an article in Science on "Enzymes in Coherent Motion."

Specifically, we have made progress in the following aspect:

(1) There are important and unprecedented properties that are likely already existed in the protein materials but are lost in the spatial and/or temporal fluctuation background;
(2) The hidden properties cannot be observed by ensemble-averaged measurements, and single-molecule measurements are able to reveal the lost properties;
(3) The hidden properties appear intermittently, and their appearances can be recorded by single-molecule property time trajectory recording and identified by our novel statistic analysis (2D Regional Correlation Analysis of Single-Molecule Time Trajectories);
(4) We have observed intermittently bunched and coherent protein conformational motions that involved in the enzymatic reaction activities. The specific conformational motions are along the nuclear coordinate controlling and regulating the substrate-enzyme active complex formation and product releasing. The critical protein conformational motions show bunched and coherent dynamics.
(5) Identifying the stimulating parameters and using novel technical approaches (such as the Atomic Force Microscopy and Magnetic Tweezers), we will be able to bring the lost properties out of the stochastic fluctuation background through temporal and spatial bunching and oscillatory synchronizations. In this way, the lost unprecedented properties will presented as an ensemble-averaged properties-showing significant impact in material bulk scale.

Technology Transfer

Final Report (Aug. 1, 2008-July 31, 2012)

Controlling Protein Conformations to Explore Unprecedented Material Properties by Single-Molecule Surgery

Grant No. W911NF-08-1-0349

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Proposal Number: 54570-MS

Abstract

Basic material research has been traditionally conducted at an ensemble-averaged level to create and manipulate a bulk material with millions of molecules, the so-called top-down approach. The complexity of molecular interaction and the inherent inhomogeneity of the molecular structure often hinder the efficiency and specificity in the exploration of novel material properties. The primary objective of our single-molecule material science project is to mechanically and optically control and regulate single-molecule protein conformations to explore unprecedented properties and capture such exclusive states in real-time at an extreme molecular sensitivity. Bring up never-detected and novel properties from proteins, over the last year, we have (1) revealed a time bunching effect of protein conformational motions under enzymatic reactions, which suggests that new material properties can be selectively induced and intensified under specific conditions identified by single-molecule experiments. The unprecedented properties can only be observed and studied by our single-molecule approaches; (2) discovered, for the first time, oscillatory enzyme conformational motions that are critical for the enzyme activity, which shed light on developing new material technology to induce, intensify, and lock random and transient material properties; (3) demonstrated a manipulation of protein conformations by force pulling a specific residue of a target protein, achieving a new technical milestone towards our goal of manipulating single-molecule proteins to create new material properties; (4) explore matrix magnetic tweezers manipulation of protein activities in enzymatic reaction and protein sensing; and (5) identified a generally existed, but hidden for conventional measurements, time bunching effect of protein conformational motions under enzymatic reactions. Our finding suggests that new material properties can be selectively induced and intensified under specific conditions identified by single-molecule experiments, which is a novel concept for exploring new material properties. The unprecedented properties can only be observed and studied by our single-molecule approaches.

Summary of the Results and Progress

The primary aim of our single-molecule material science project is to bring up never-detected and unprecedented properties from proteins. If a random and transient (fluctuating) material property, typically non-detectable and lost as it is spatially and temporally averaged out, can be induced to appear at a specific temporal or frequency domain, then a novel property is created from the protein material. During the four years of the project, we have made significant progresses towards realizing the aim of our project. Specifically, we have made discoveries of a novel bunching effect and a coherence dynamics of protein conformational properties that are critical for enzyme functions. We have provided a novel approach to create unprecedented material properties by inducing the specific properties bunched and/or

coherence in time. We have also made a significant technical development on demonstrating a manipulation of protein conformations by force pulling a specific residue of a protein, accomplishing a technical milestone of manipulating single-molecule proteins to create new material properties. We have already published a number of papers to report our discoveries, including a recent article published in *J. AM. Chem. Soc.* on "Probing Single-Molecule Enzyme Active-Site Conformational State Intermittent Coherence," and an article in *Science* on "Enzymes in Coherent Motion."

Specifically, we have made progress in the following aspect:

(1) There are important and unprecedented properties that are likely already existed in the protein materials but are lost in the spatial and/or temporal fluctuation background;
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(3) The hidden properties appear intermittently, and their appearances can be recorded by single-molecule property time trajectory recording and identified by our novel statistic analysis (2D Regional Correlation Analysis of Single-Molecule Time Trajectories);
(4) We have observed intermittently bunched and coherent protein conformational motions that involved in the enzymatic reaction activities. The specific conformational motions are along the nuclear coordinate controlling and regulating the substrate-enzyme active complex formation and product releasing. The critical protein conformational motions show bunched and coherent dynamics.
(5) Identifying the stimulating parameters and using novel technical approaches (such as the Atomic Force Microscopy and Magnetic Tweezers), we will be able to bring the lost properties out of the stochastic fluctuation background through temporal and spatial bunching and oscillatory synchronizations. In this way, the lost unprecedented properties will presented as an ensemble-averaged properties-showing significant impact in material bulk scale.

A.Time Bunching Effect of Single-Molecule Conformational Dynamics in Enzymatic Reactions. Typically, protein conformational motions are random and controlled by thermal fluctuations and local environment fluctuations asscociated with electric static fields and molecular interactions. Bunching effect, a conformational motion time being non-randomly distributed (bounched together) in a specific time scale, has been revealed and identified to be associated with substrate-enzyme complex formation in T4 lysozyme conformational dynamics under enzymatic reactions. Using single-molecule fluorescence spectroscopy, T4 lysozyme conformational motions under the hydrolysis reaction of polysaccharide were probed by monitoring the fluorescence resonant energy transfer (FRET) between a donor-acceptor probe pair tethered to T4 lysozyme domains involving open-close hinge-bending motions.

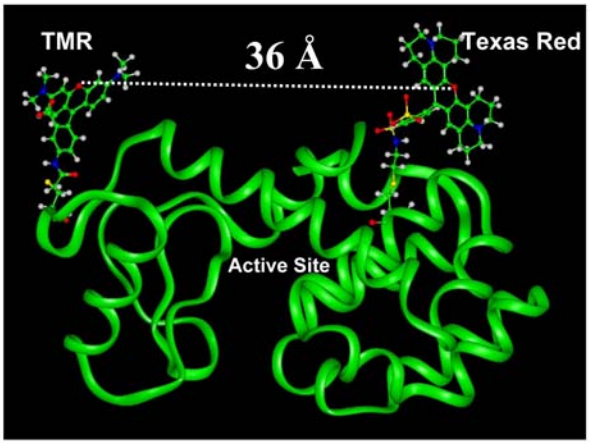

Figure 1. Crystal structure of the wild-type T4 lysozyme (3LZM, from the Protein Data Bank). A pair of

tetramethylrohdamine and Texas Red dye molecules is covalently tethered to the cysteine 54 and cysteine 97 residues of a T4 lysozyme protein molecule as donor and acceptor for FRET measurement.

Bunching effect, implying that conformational motion times tend to bunch in a finite and narrow time window, presents non-random molecular conformational motions. Bunching effect has never been observed previously for molecular conformational motions under enzymatic reactions. In this ARO project, we have observed and identified an unprecedented bunching effect associated with substrate-enzyme complex formation in T4 lysozyme conformational dynamics under enzymatic reactions (Figures 2 and 3). Using single-molecule fluorescence spectroscopy, we have probed T4 lysozyme conformational motions under the hydrolysis reaction of polysaccharide of *E. coli B* cell walls by monitoring the FRET between a donor-acceptor probe pair tethered to T4 lysozyme domains involving open-close hinge-bending motions. We have identified the bunching effect of the substrate-enzyme active complex formation time in T4 lysozyme enzymatic reactions. We show that the bunching effect, a dynamic behavior observed for the catalytic hinge-bending conformational motions of T4 lysozyme, is a convoluted outcome of multiple consecutive Poisson rate processes that are defined by protein functional motions under substrate-enzyme interactions; i.e., convoluted multiple Poisson rate processes give rise to the bunching effect in the enzymatic reaction dynamics. We suggest that the bunching effect is likely common in protein conformational dynamics involving in conformation-gated protein functions.

Bunching effect in single-molecule T4 Lysozyme non-equilibrium conformational dynamics under enzymatic reactions

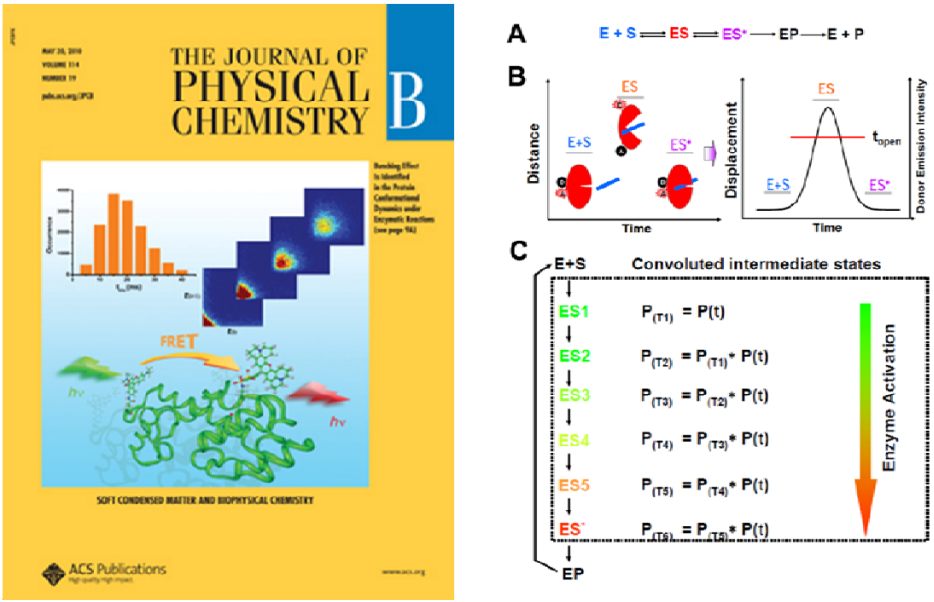

Figure 2. (Left) The cover page highlighted our work reporting the discovery of bunching effect in enzymatic protein conformational motions under enzymatic reactions. A pair of

tetramethylrohdamine and Texas Red dye molecules is covalently tethered to the cysteine 54 and cysteine 97 residues of a T4 lysozyme protein molecule as donor and acceptor for FRET measurement. A modified Mechaelis-Menton mechanism for T4 lysozyme conformational dynamics. **(A)** Schematic presentation of the enzymatic reaction. E, S, and P represent the enzyme, substrate, and product, respectively. **(B)** Correlation between the enzyme hinge-bending motion and the variation of donor fluorescence intensity in one ES*-formation time. **(C)** Scheme of the enzyme reaction and the formation-time probabilities of multiple convoluted intermediate states. From E+S to ES^*, the enzyme experiences multiple intermediate states and finally reaches the active state, ES^*. *P(t)* is the probability distribution of the step times. $P_{(Tn)}$ (n=1, 2, 3, 4, 5 6) are the formation-time probabilities for the intermediate states, and ES^* is readied for the enzymatic reaction.

The conformational dynamics is significantly regulated by the interactions between the T4 lysozyme and the substrate in terms of electrostatic attraction and hydrogen bonding interactions, being associated with the formation of the active complex state of ES* (Figure 2) for the T4 lysozyme enzymatic reactions. For the formation of the first intermediate state, the distribution of the conformational motion time is stochastic and there is no bunching among the conformational motion times. However, with subsequent intermediate states being formed in consecutive multiple steps, the bunching effect appears for the formation times and in turn becomes more and more prominent. It is most likely that the bunching effect is a general property for the conformational dynamics of enzymes that regulated and rate-limited by conformational motions in forming enzyme-substrate complex states. Typically, functional conformation selection mechanism applies when bunching effects in the conformational dynamics existed.

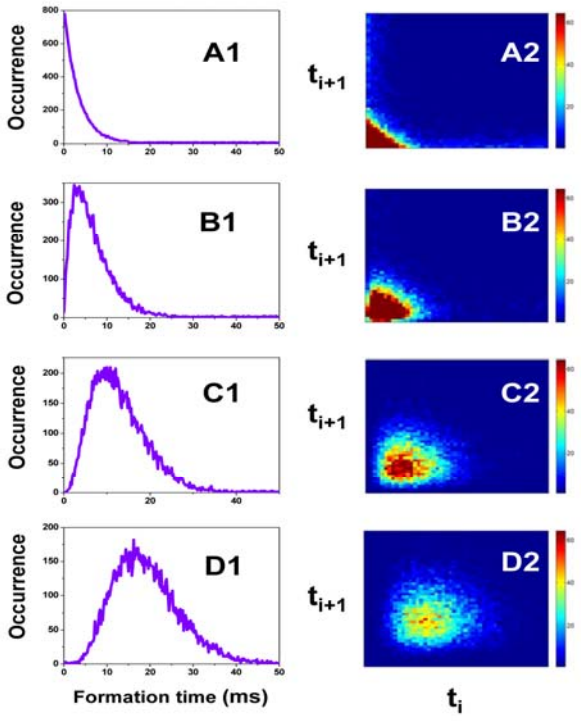

Figure 3. Simulated distribution of formation times (A1 to D1) and corresponding two dimensional joint probability distributions (A2 to D2) of adjacent formation times for different intermediate steps. As a single step, A1 shows exponential distribution and A2 only shows a wing structure implying one step is a Poisson process and there is no bunching effect. However, for the multiple steps such as 2, 4, 6 steps, non-exponential distributions of the probabilities (in B1, C1 and D1) and the bunching structures (in B2, C2 and D2) are increasingly clear, implying the bunching nature in the open-close conformational motion times.

In a Poisson rate process, there should be no bunching among the stochastic conformational fluctuation times. However, a non-equilibrium rate process through a sequence of consecutive Poisson processes with comparable rates, eventually produces a bunching effect within the overall time lapse for the overall multiple-step rate process. For T4 lysozyme's conformational dynamics, the physical nature of the bunching effect is associated with the functionally-conformational motion mechanism involving in non-equilibrium conformational fluctuations. The characteristics of the non-equilibrium conformational fluctuation dynamics is experimentally-observed by oscillatory fluctuations of the conformational open-close motions and the Gaussian-like formation time distributions.

B. Revealing Single-Molecule Enzyme Active-Site Conformational Change Coherence. Using single-molecule fluorescence resonance energy transfer (FRET), we revealed oscillatory conformational changes in a kinase protein, 6-Hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPK) (Figures 2 and 3), involving substrate binding and product releasing in enzymatic reaction turnovers. It has long been anticipated theoretically that the protein conformational oscillations may couple with the enzymatic reaction conformational dynamics, which cannot be observed by ensemble-averaged experiments since that the appearances of possible oscillatory conformational motions are unsynchronized from molecule to molecule. Our observation is obtained through a systematic single-molecule assay and fluctuation time-trajectory recording experiments under various enzymatic reaction conditions, providing utilizing correlation function analyses and our newly-developed 2-D correlation function amplitude distribution analysis.

In this experiment, we covalently attached two fluorophores, Cy3 (donor) and Cy5 (acceptor) to the lid 2 and the core of the HPPK protein molecules, respectively (Figure 4A).

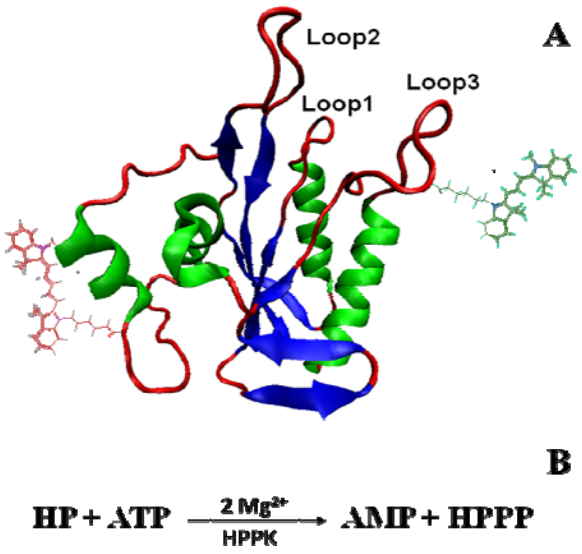

Figure 4. (A) Overall Structure of HPP enzyme. The cyan spirals represent the α helices and the green arrows are the β strands. The loops are shown by the pipes. Amino acid residue 88 has been labeled with Cy3 (donor, blue) and residue 142 is labeled with Cy5 (acceptor, red). There are three flexible loops of the HPPK involve in the enzymatic conformational changes critical to the enzymatic reaction activity. Among the three loops present in HPPK, loop 3 undergoes the most dramatic open-close conformational changes in each catalytic cycle, correlating with substrates (HP and ATP) binding. The three flexible lids of the HPPK involve in the enzymatic active-site conformational changes critical to the enzymatic reaction activity. Among the three lids present in HPPK, both lid 2 and lid 3 undergo significant open-close conformational changes in each catalytic cycle, correlating with substrates (HP and ATP) binding. **(B)** Sequential Reaction Mechanism of HPPK-Catalyzed Pyrophosphoryl Transfer. The binding of the substrate HP is facilitated in the presence of MgATP.

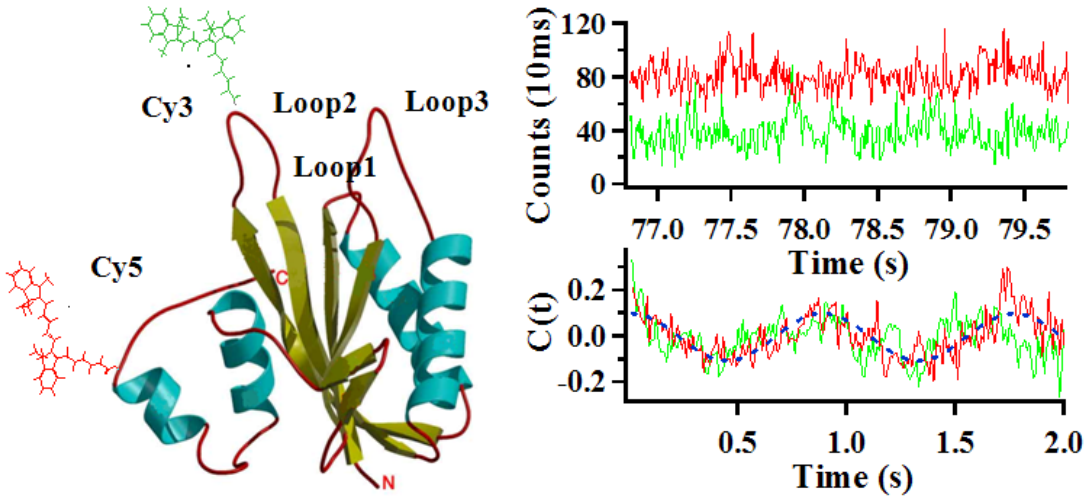

Figure 5. (Left) Overall Structure of HPPK enzyme. The cyan spirals represent the α helices and the green arrows are the β strands. The loops are shown by the pipes. Amino acid residue 88 is labeled with Cy3 (donor, blue) and residue 142 is labeled with Cy5 (acceptor, red) on loop 2. There are three flexible loops of the HPPK involve in the enzymatic conformational changes critical to the enzymatic reaction activity. The three flexible loops of the HPPK involve in the enzymatic active-site conformational changes critical to the enzymatic reaction activity. Among the three loops present in HPPK, both loop 2 and loop 3 undergo significant open-close conformational changes in each catalytic cycle, correlating with substrates (HP and ATP) binding. **(Right)** A pair of typical FRET time trajectories and its correlation function (C(t)) revealing the oscillatory behavior originated from the coherence in the enzyme active-site conformational state changes.

Although, these types of single-molecule coherence have been intensively debated and anticipated by theoretical literature, such dynamic behaviour has never been experimentally recorded before. Previously, none of the single-molecule spectroscopy work published on enzymes (including a few recent publications on other single-molecule kinases) conformational dynamics has reported these types of coherent dynamics. Throughout the process of enzymatic reaction, the individual conformers are not static; rather there exist dynamic fluctuations among the various forms. According to our single-molecule FRET measurements, the open and close of the loops 3 and 2 of HPPK, are induced by substrates, and the entire enzymatic reaction is highly dynamic in nature where the catalytic loops undergo opening up and closing down in time scales of milliseconds. The interplay between the protein structures, folding/unfolding-function internal relationships resulting in opening and closing of the active loops of the enzyme.

The recording of the coherent conformational dynamics provides a real-time observation of single-molecule protein dynamics under non-equilibrium conditions in enzymatic reactions. Typically, an enzymatic reaction involves a complex substrate-active site interaction, protein domain disorder-to-ordered (unfolded-to-folded or flexible-to-rigid) conformation transition, active substrate-enzyme complex formation, chemical transformation, and product releasing. The dynamic and complex process provides a recurrence of changes of the protein conformations at the active site from disordered to ordered coherent changes driven by substrate interaction and binding as well as by product releasing. The conformational recurrence is driven by recurrence of the electrostatic field at the active site, the electrostatic interactions between the substrate and the active site of the enzyme, and the local environment of the enzymatic active site. It is the substrate binding and forming the substrate-enzyme complex serve as a negative enthalpy source

for the oscillatory dynamics at the single-molecule level. Damping at a time scale of dephasing due to local environment fluctuations and multiple nuclear coordinate fluctuations dictates the resolved conformational oscillation coherence and brings up the intermittent appearances of the oscillation correlation observed.

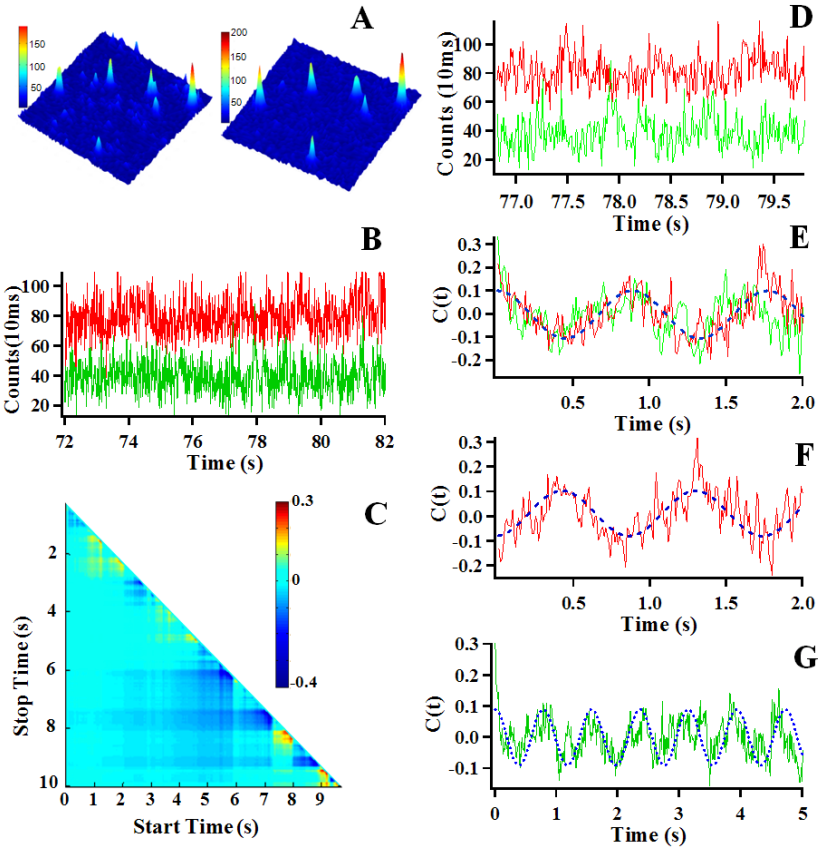

Figure 6. (A) Single molecule fluorescence images (10μm×10μm) of Cy3 and Cy5 labeled HPPK in the presence of 100 μM ATP and 100 μM HP. The emission is from the Cy3 (Left) and Cy5 (Right) dyes attached to the HPPK enzyme. **(B)** A part of the single-molecule intensity-time trajectories of the donor (Cy3, Green) and acceptor (Cy5, Red) labeled on HPPK in the presence of 100 μM ATP and 100 μM HP. **(C)** The result of TCAD analysis on the single-molecule donor-acceptor fluorescence intensity trajectories shown in 3B. The cold color represents that the D-A is anti-correlated, whereas the warm color represents that the D-A is correlated. **(D)** A part of the single-molecule intensity-time trajectories of the donor and acceptor from the long trajectories shown in 3B, the anti-correlated fluctuation features are evident. **(E)** Autocorrelation functions *($C(t)_{AA}$ and $C(t)_{DD}$)* of the donor (Green) and the acceptor (Red) from the single-molecule intensity-time trajectories shown in 3D, the fitted (blue) oscillatory frequency is 1.2±0.1 s^{-1} **(F)** Cross-correlation function ($C(t)_{AD}$) from the single-molecule intensity-time trajectories shown in 3D, the fitted (blue) coherence frequency is 1.2±0.1 s^{-1}, the same as $C(t)_{AA}$ *and* $C(t)_{DD}$. The result of the same decay rates of the three correlation functions calculated from the single-molecule D-A single time trajectories indicates that the fluctuation dynamics is originated from the same origin, i.e., single-molecule FRET fluctuations associated with conformational change fluctuations of HPPK. **(G)** Autocorrelation $C(t)_{DD}$ of the donor (Green) from the single molecule intensity-time trajectories shown in 3B, dephasing appears in ~4.5 seconds.

C. Manipulation of Single-Molecule Protein Conformations by AFM-FRET Ultramicroscopy. Protein conformations play crucial roles in enzyme functions and protein-protein interactions. Dynamic views of protein-folding and conformational changes are critical for understanding protein functions. For example, an enzyme protein can have different activities under different conformations. Also, protein-protein recognition involves fluctuating protein conformations and folding-binding cooperative interactions, conformational changes can significantly change the affinity and selectivity of the protein interaction and binding properties, which in turn often contribute to dramatic changes in protein functions. In recent years, more and more biological research works have identified that even genetically identical proteins under the same physiological conditions can have dramatically different functions and activities at different time and different location in living cells, best described as static and dynamic disorders. The overarching parameter that gives the disorders is the inhomogeneous and ever changing conformations of the proteins. There is a solid foundation to support that the most important and effective parameter to manipulate protein functions is to control or manipulate protein conformations.

Mechanical force manipulations of protein conformations do change protein activity significantly. Both theoretical and experimental demonstrations suggest that AFM tip control protein conformations can definitely manipulate protein activities. For example, AFM mechanical controlling protein conformation can change not only the kinetics but also the pathway of a disulfide bond reduction activity. Also, it has been theoretically demonstrated that oscillating force applied on an enzyme protein at the frequency of enzymatic reaction turnover rate enhances the enzymatic reaction activities significantly due to force modification of the reaction pathway, potential surface, and enzymatic reaction intermediate state energy.

We have demonstrated technically a new single-molecule material manipulation approach of a new AFM-FRET single-molecule imaging and manipulating microscope. With this new technique, we were capable of pulling a single protein molecule from its native folded conformation to various controlled unfolded conformations at a specific amino acid residue (Figure 7). In this work, we chemically linked a single protein molecule to surface at specific position so that we can pull single protein molecule at a specific conformational coordinate (Figure 7). We have recently used the AFM-FRET ultramicroscopy to demonstrate simultaneous measurements of force spectroscopy and fluorescence resonance energy transfer (FRET) during a single-molecule pulling event of a kinase enzyme (HPPK). By analyzing the time resolved FRET trajectory and the correlated force spectroscopy, we reveal the correlation between force perturbation and conformation change of enzyme. Further, we demonstrated the possibility of single-molecule spectroscopy detection under force perturbation.

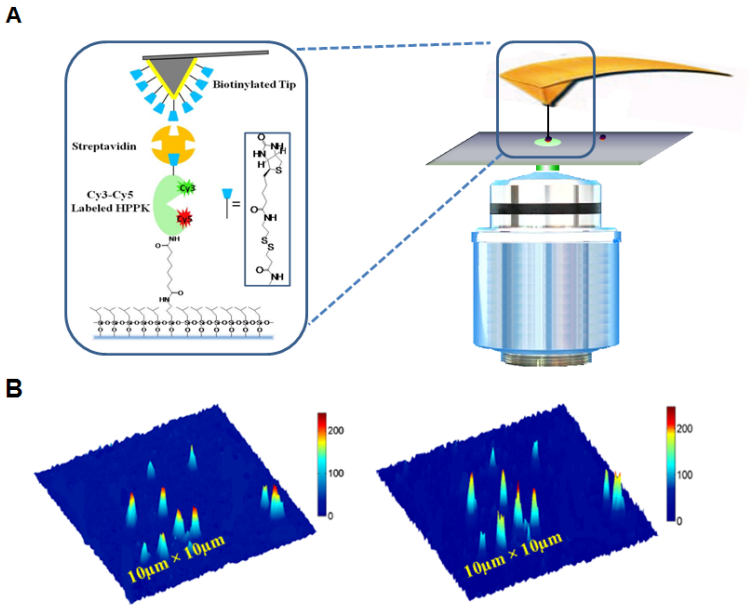

Figure 7. **(A)** Single-molecule AFM-FRET ultra microscopy, the zoomed panel in the left presents schematic diagram of one FRET dyes (Donor-Acceptor: Cy3-Cy5) pair labeled HPPK molecule tethered between a glass cover-slip surface and a handle (biotin group plus streptavidin), and a biotin group modified AFM tip. **(B)** Single-molecule fluorescence photon counting images of the donor (Cy3, left) and accepter (Cy5, right). Each feature is from a single HPPK enzyme labeled with Cy3-Cy5 FRET dyes.

We demonstrated simultaneous single-molecule protein measurements of force spectroscopy and FRET spectroscopy in single molecule pulling events, using FRET labeled enzyme (HPPK kinase) proteins as a model system. By recording and analyzing single-molecule FRET photon-counting time trajectories and force spectroscopy, we demonstrated a series of new correlated single-molecule measurements of an enzyme protein conformation changes under AFM tip force pulling and manipulation. In our experiment, we were able to co-axle position the AFM tip with a target single molecule within about 1 nm, and carry out an AFM tip force pulling of a single enzyme and recording of single-molecule FRET signal simultaneously. We identify single target molecule position by direct AFM scanning, which establishes a new stage for manipulating protein conformation and simultaneously monitoring the protein function changes for controlling the conformation of enzyme under an enzymatic reaction. This method will be very useful for analyzing protein structure and dynamics, and it is now possible to study real-time manipulated protein-ligand interactions and enzymatic reactions when a single biomolecule is performing a biological function.

In this work, we chemically linked a single protein molecule to surface at specific position so that we can pull single protein molecule at a specific conformational coordinate (Figure 7 and 8B). We have used the AFM-FRET ultramicroscopy to demonstrate simultaneous measurements of force spectroscopy and fluorescence resonance energy transfer (FRET) during a single-molecule pulling event of HPPK (Figure 8). By analyzing the time resolved FRET trajectory and the correlated force spectroscopy, we reveal the correlation between force perturbation and conformation change of enzyme. Further, we demonstrated the possibility of single-molecule spectroscopy detection under force perturbation.

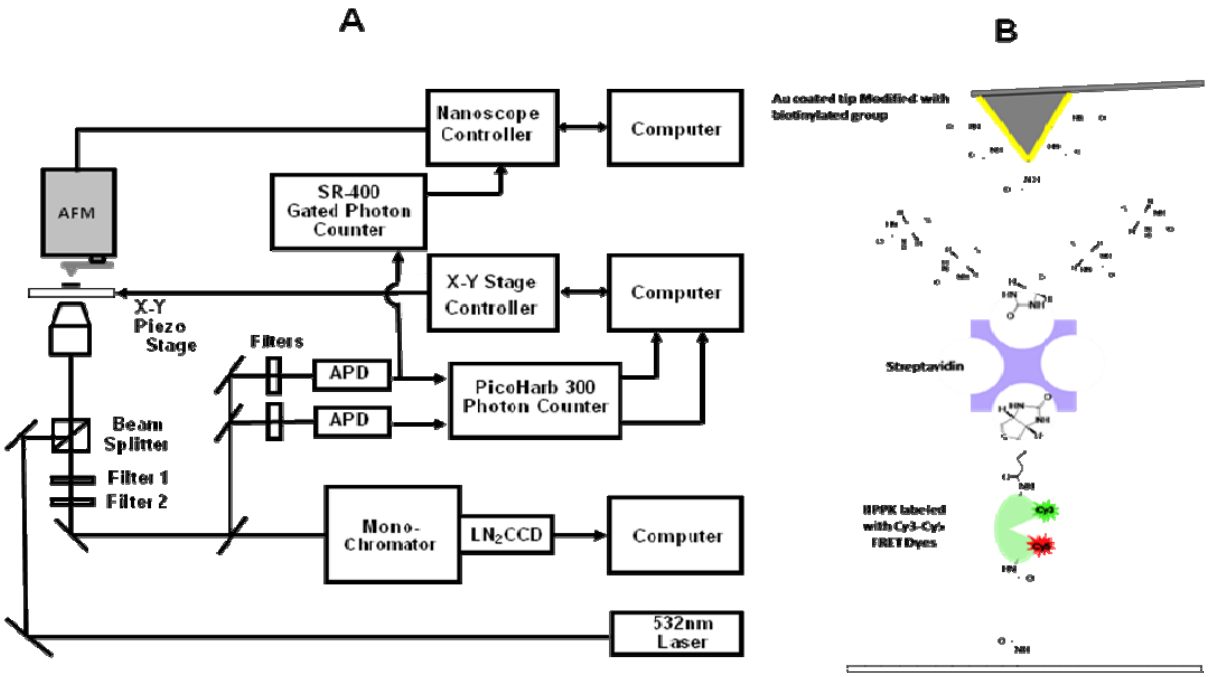

Figure 8. Atomic-force-pulling manipulation of the conformations of a single target molecule.

We demonstrated simultaneous single-molecule protein measurements of force spectroscopy and FRET in single molecule pulling events, using FRET labeled HPPK proteins as a model system. By recording and analyzing single-molecule FRET photon-counting time trajectories and force spectroscopy, we demonstrate a series of new correlated single-molecule measurements of an enzyme protein conformation changes under AFM tip force pulling and manipulation (Figure 9).

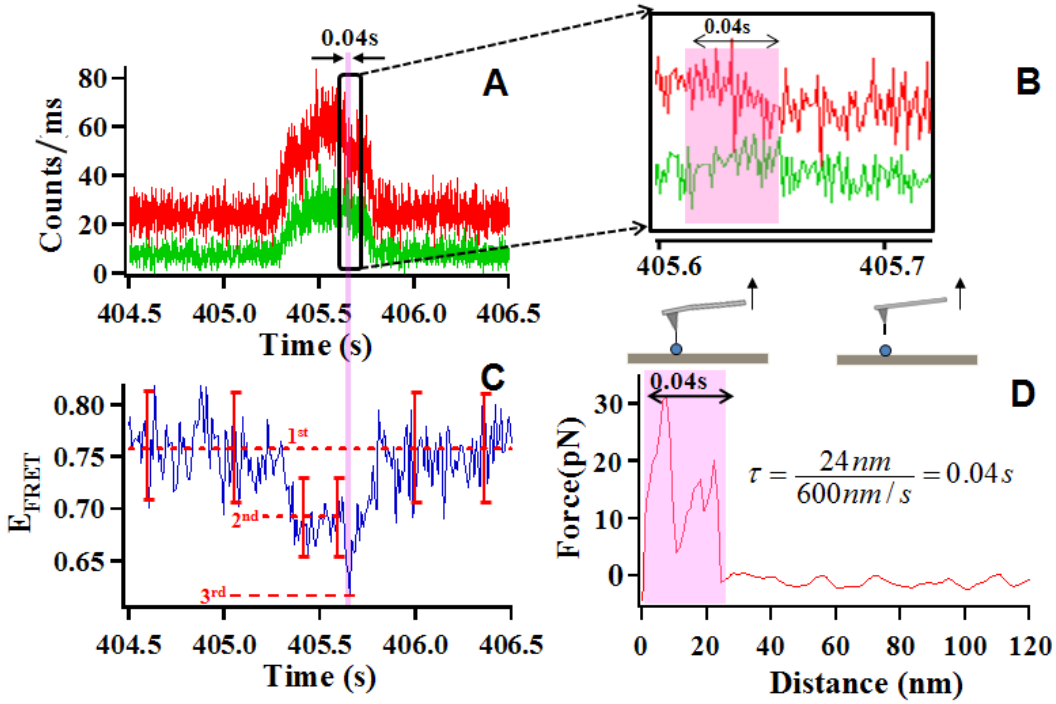

Figure 9. **(A)** A typical FRET time trajectory of donor (green) and acceptor (red) associated with one single-molecule AFM-FRET force pulling event. **(B)** Zoom-in intensity trajectory of donor and acceptor from (A), the highlighted intensity change is correlated to one pulling event occurred in 0.04s. **(C)** FRET efficiency time trajectory of one single-molecule AFM-FRET pulling event, in the whole process of AFM tip travelling route from approaching the protein from far away to moving away out of the micro-mirror effect distance range, three efficiency levels are recorded and identified. The error bar shows the ±2SD (standard deviation) indicating ≥ 95% precision of identification of the data points within the range. **(D)** The correlated force curve, the curve shows the extension length of 24 nm within a period of 0.04 s.

The results (Figure 10) show a number of significant characteristics: (1) There are multiple peaks appearing in single domain from unfolding a single protein molecule. The multiple peaks in the single-molecule pulling force spectroscopy are primarily attributed to the traces associated with unfolding of the single segments, loops, or domains. The data are also associated with fluctuations due to the rugged landscape of protein folding with multiple local minima. For example, in Figure 10A, the force pulling curve shows two small peaks that come from the unfolding single protein domain DomC between residue 119 and residue 142, which suggests that our AFM-FRET nanoscopy approach is capable of probing the substructures of the protein domain from force curves. Although, at this stage, we are not able to identify each peak with the exact fragment in the protein domains, this observation of substructures in single molecule force spectroscopy is highly promising, which allows AFM force pulling to be a potentially powerful tool for offering insight into the details of the protein domains; (2) In the single molecule force curve, the order of these peaks did not follow the protein structure sequence order (Figure 5E), indicating the order of the rupture did not follow the exact pattern of protein substructure sequence order in the molecule. For example, the observed order of the peaks in Figure 10A (DomC) or 10B (DomB and DomC) did not show the same order as they appeared in Figure 10C

(DomA, DomB and DomC). We attributed this order variance to the different overall affinity among amino acid residues in substructures to resist pulling force, resulting in that some of the substructures are easier to be unfolded or ruptured, while others are not. We also attributed this result to cooperative unfolding nature of three domain; individual domains were not unfolded independently when not just one domain was stretched, as described in Figure 10B and 10C. (3) The force for rupturing a single protein molecule is small; the range of the force distribution is between 5 to 20 pN, as shown in the three force curves.

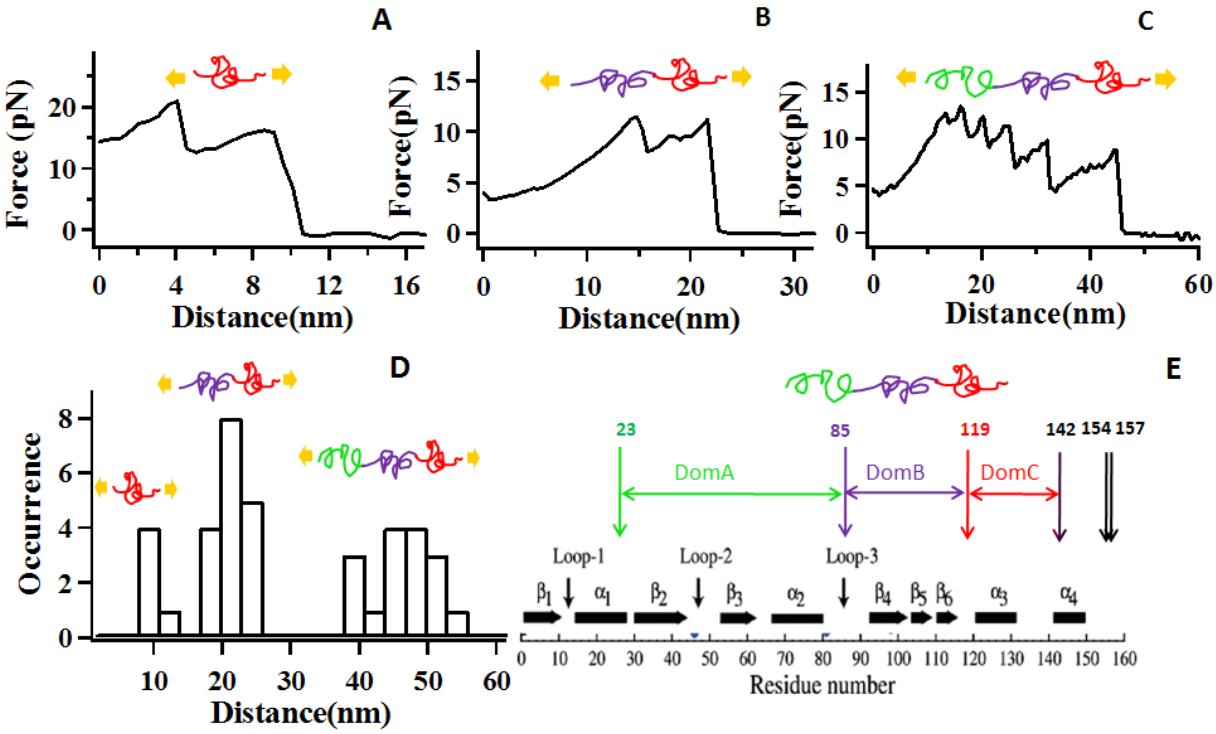

Figure 10. (A-C) Three types of single-molecule force pulling curves of HPPK, as HPPK was chemically linked to a glass coverslip at residue 142, and AFM tip pulling occurs at the possible lysine residue site 119, 85, and 23. In the insets above the force curves, three proposed domains are colored (green for DomA, purple for DomB and red for DomC) and depicted. (A) The unfolding force curve of DomC (red), which corresponds to the rupture distance 9 nm. (B) The unfolding force curves of DomB (purple) and DomC (red), corresponding to the rupture distance 22 nm. (C) The unfolding force curve of DoA, DoB and DoC, and the rupture distance is 45 nm. **(D)** Histogram of protein rupture distance distribution. The distribution of the rupture distances shows three peaks, at about 9 nm (DomC), 22 nm (DomB and DomC), and 45 nm (DomA, DomB and Dom C). (E) The structure of the HPPK mutant (the site of lysines and cysteine are illustrated). Amino acid residue 142 was mutated to cysteine for specific site tethering of HPPK on the glass cover-slip.

Overall, we have demonstrated a novel approach of single-molecule AFM-FRET nanoscopy that is capable of conducting simultaneous single-molecule force manipulation and FRET measurement for a targeted single protein molecule. Using this approach, we are able (1) to

locate an individual Cy3-Cy5 labeled enzyme molecule in a pinpoint nanoscale precision; (2) to force pulling and unfolding the target single enzyme molecule; and (3) to simultaneously probe the protein conformational changes by single-molecule FRET spectroscopy measurement during the AFM pulling event. Our demonstrated single-molecule AFM-FRET nanoscopy presents a novel approach of studying protein structure-function dynamics and mechanism. Using the nanoscope, we have specifically demonstrated the force pulling manipulation of a kinase enzyme and simultaneously probed the manipulated conformational changes by correlated single-molecule FRET recording, which showed multiple rupture coordinates in single-molecule enzyme force unfolding processes. The AFM-FRET nanoscopy provides a new approach of analyzing the landscape of protein folding and manipulating protein conformations to explore new properties.

Balanced Budget:

- Lu's project (Grant No. W911NF-08-1-0349) has already submitted the final financial report by June 2012, and the report has been accepted by ARO financial office.

Publications of ARO sponsored research (Aug. 2008-July 2012)

1. Yufan He, Maolin Lu, Jin Cao, H. Peter Lu, "Manipulating Protein Conformations by Single-Molecule AFM-FRET Nanoscopy," ACS Nano, **6**, 1221-1229 (2012).

2. H. Peter Lu, "Enzymes in Coherent Motion," *Science*, **335**, 300-301 (2012).

3. Desheng Zheng, Leonora Kaldaras, H. Peter Lu, "Total Internal Reflection Fluorescence Microscopy Imaging-Guided Confocal Single-Molecule Fluorescence Spectroscopy," *Review of Scientific Instruments*, **83**, 013110 (2012).

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5. Suraj Saraswat, Anil Desireddy, Desheng Zheng, Lijun Guo, H. Peter Lu, Terry P. Bigioni, and Dragan Isailovic, "Energy Transfer from Fluorescent Proteins to Metal Nanoparticles," *J. Phys. Chem. C* **115(35)**, 17587-17593 (2011).

6. H. Peter Lu, "Revealing Time Bunching Effect in Single-Molecule Enzyme Conformational Dynamics," *Phys. Chem. Chem. Phys.*, **13**, 6734-6749 (2011).

7. Sara M. Belchik, David W. Kennedy, Alice C. Dohnalkova, Yuanmin Wang, Papatya C. Sevinc, Hong Wu, Yuehe Lin, H. Peter Lu, James K. Fredrickson, and Liang Shi, "Extracellular Reduction of Hexavalent Chromium by MtrC and OmcA of Shewanella oneidensis MR-1," *Applied and Environmental Microbiology, 77, 4035-4041* (2011).

8. Yuanmin Wang, H. Peter Lu, "Bunching Effect in Single-Molecule T4 Lysozyme Non-Equilibrium Conformational Dynamics under Enzymatic Reactions," *J. Phys. Chem. B*, **114**, 6669-6674 (2010) (Cover page).

9. H. Peter Lu, "Acquiring a Nano-View of Single Molecules in Actions," *Nano Reviews* **1**, 6-7 (2010).

10. H. Peter Lu, "Single-Molecule Protein Conformational Dynamics in Enzymatic Reactions," Chapter 24, PP471-494 in Single Molecule Spectroscopy in Chemistry, Physics and Biology: Nobel Symposium (Springer Publishing, 2010).

11. Yufan He, Xiaohua Zeng, Saptarshi Mukherjee, Suneth Rajapaksha, Samuel Kaplan, H. Peter Lu, "Revealing Linear Aggregates of Light Harvesting Antenna Proteins in Photosynthetic Membranes," *Langmuir* **26**, 307-313 (2010) (Cover page).

12. H. Peter Lu, "Single-Molecule Protein Interaction Conformational Dynamics," Current Pharmaceutical Biotechnology, **10**, 522-531 (2009).

Invited talks of ARO sponsored research (Aug. 2008-Jul. 2012)

H. P. Lu, "Single-Molecule Protein Conformational Dynamics in Enzymatic Reactions and Cell Signaling," Medical School and Department of Chemistry, University of Southern California, Los Angeles, Sept. 25-28, 2008.

H. P. Lu, "Single-Molecule Protein Conformational Dynamics in Enzymatic Reactions and Cell Signaling," Department of Chemistry, Boston College, Boston, Nov. 5, 2008.

H. P. Lu, "Probing Single-Molecule Protein Conformational Dynamics in Enzymatic Reactions," Single-Molecule Biophysics Meeting, Aspen, CO, Jan. 4-10, 2009.

H. P. Lu, "Single-Molecule Protein Conformational Dynamics," Department of Physics and Department of Chemistry, Arizona State University, Tempe, Apr. 8, 2009.

H. P. Lu, "Single-Molecule Protein Dynamics in Enzymatic Reactions and Cell Signaling," Single Molecule Dynamics International Workshop, Telluride, CO, June, 22-26, 2009.

H. P. Lu, "Revealing Bunching and Memory Effects in Single-Molecule Protein Conformational Dynamics under Enzymatic Reactions," the 239th ACS National Meeting, San Francisco, Mar. 21-25, 2010.

H. P. Lu, "Manipulating Single-Molecule Protein Conformations for Novel Dynamics," Single Molecule Dynamics International Workshop, Telluride, CO, June, 13-17, 2010.

H. P. Lu, "Revealing Bunching and Memory Effects in Single-Molecule Protein Conformational Dynamics under Enzymatic Reactions," the 240th ACS National Meeting, Boston, Aug. 22-26, 2010.

H. P. Lu, "Revealing Bunching and Memory Effects in Single-Molecule Protein Conformational Dynamics under Enzymatic Reactions," the 242th ACS National Meeting, Denver, Aug. 27-31, 2011.

H. P. Lu, "Probing Single-Molecule Enzyme Active-Site Conformational State Intermittent Coherence and Time Bunching Dynamics," the 243th ACS National Meeting, San Diego, Mar. 25-29, 2012.

H. P. Lu, "Probing Single-Molecule Enzyme Active-Site Conformational State Intermittent Coherence and Time Bunching Dynamics," the APS Regional Meeting, Columbus, Apr. 13-14, 2012.

H. P. Lu, "Probing Single-Molecule Enzyme Active-Site Conformational State Intermittent Coherence and Time Bunching Dynamics," Characterizing Landscapes: From Biomolecules to Cellular Networks International Workshop, Telluride, CO, June, 11-15, 2012.

Student Metrics for this Reporting Period (Aug. 2008-July 2012)

Graduate student: Suneth Rajapaksha, 50% FTE, GPA 3.33
Postdoctoral Fellow: Dr. Yufan He, 50% FTE
Postdoctoral Fellow: Dr. Yuanmin Wang, 20% FTE
Postdoctoral Fellow: Dr. Desheng Zheng, 30% FTE
Faculty: PI, Dr. H. Peter Lu, 0.75 summer month support

One student graduated during this funding period.